Investigation of *Saccharomyces cerevisiae* Trm10 tRNA methyltransferase (m^1G_9) activity: Substrate Specificity and Essential Amino Acid Residues for Catalysis

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By

Jeremy Henderson

The Ohio State University

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Project Advisor: Dr. Jane E. Jackman, Department of Biochemistry

Abstract

Trm10 catalyzes *N-1* methylation of G_9 (m^1G_9) for at least 10 tRNA species in *S. cerevisiae,* including $tRNA^{Gly}$. However, at least 12 yeast tRNA species, including $tRNA^{Val}$ and $tRNA^{Leu}$, have an unmodified G_9, and the sequence elements that define tRNA substrates for Trm10 are unknown and are not obvious from simple sequence comparison. To investigate Trm10 tRNA substrate specificity, we have developed a sensitive assay using site-specifically labeled Gly, Leu and Val tRNAs. Consistent with the in vivo methylation pattern, $tRNA^{Leu}$ is not a Trm10 substrate, yet surprisingly, both $tRNA^{Gly}$ and $tRNA^{Val}$ are substrates for m^1G_9 modification in vitro. A comparison of steady-state kinetic parameters for Trm10 activity with $tRNA^{Val}$ and $tRNA^{Gly}$ reveals that k_{cat}/K_M, an indicator of substrate specificity, is nearly identical for both substrates. Therefore, these data suggest that Trm10 must use additional mechanisms to distinguish between some tRNA substrates in vivo.

Since the mechanism by which Trm10 methylates tRNA is unknown and there is no identifiable sequence homology between Trm10 and other methyltransferases, it is important to identify Trm10 residues that participate in catalysis. We have investigated the role of several highly conserved Trm10 residues by mutagenesis of these residues to alanine, revealing at least 8 amino acids that appear to be important for catalysis, based on significantly decreased methylation activity observed for each variant, and at least 2 amino acid residues that appear to be important for substrate recognition, based on similar methylation activity of these two variants with Gly and Val tRNA substrates. The identification of these residues is a necessary and important first step toward determination of the molecular basis for Trm10 m^1G_9 activity, a potentially novel methyltransferase enzymatic mechanism.

Introduction

A tremendous amount of cellular energy is committed towards tRNA processing, editing, and modification. The simple answer for why tRNA maturation and maintenance necessitates priority in depletion of cellular energy stores is relatively obvious; tRNAs are essential adaptor molecules required for translation of genes into many of the functional and structural units of the cell, or proteins. This *biological-world*view convincingly argues that tRNAs are essential biological molecules that play a critical role in maintaining life and inherent processes; a Rosetta stone of the molecular world which functions to decode mRNA hieroglyphics coordinately with the ribosome, into vital protein products. tRNAs central role in biology is unquestionable, however this does not adequately explain the biological significance for the multitude of tRNA modifications and biogenesis steps that have hitherto escaped explanation.

Among classes of RNA molecules, tRNAs are among the most extensively modified. Nearly all tRNA molecules throughout every domain of life contain modified nucleic acids that can be methylated, which is also observed on associated ribose sugars, isoprenylated, carboxymethylated, dihydrouridylated and psuedouridylated to name a few (*1-3*). Moreover types, or subsets, of modifications are often conserved at unique positions across the spectrum of eukaryotic tRNAs. Some examples include m^1G_{37}, which ensures the fidelity of translation by prevention of a specific frameshift mutation (*4*), or $m^2{}_2G$ in yeast which occurs only at G_{26} in many tRNAs (*3*).

The locations of tRNA modifications often provide insight related to their potential biological significance and modifications can be characterized into two general categories based on this parameter. Essential modifications are often found to occur in or around the anticodon

and are critical for ensuring the fidelity of translation; m^1G_{37} is one example already mentioned that occurs next to the anticodon in the anticodon loop. The anticodon itself usually contains at least one if not more modified bases, some of which can be important for wobble pairing (*5-8*) or serve as identity markers for aminoacylation (*9*). The other category of tRNA modifications are further removed from the anticodon loop/stem. Notably, many of these have been deemed "non-essential" due to the fact that single deletion strains of the enzymes that catalyze these modifications do not exhibit observable growth defects, however a growing body of evidence indicates that these modifications may play important roles in tRNA function, perhaps by ensuring the stability of tRNAs or affecting nuclear-cytoplasmic trafficking, since combinations of two or more of these deletion mutants result in serious defects in cell growth (*10-12*).

The modification investigated in this study is *N*-1 methylation of guanosine residues (Fig.1) unique to position 9 (m^1G_9), particularly with respect to specific tRNAs found in *Saccharomyces cerevisiae*. The enzyme responsible for m^1G_9 formation is Trm10, a methyltransferase that catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to the *N*-1 atom of guanosine, resulting in the formation of m^1G and S-adenosylhomocysteine (SAHcy) as products. Before this investigation a biochemical genomics based approach was used to identify *TRM10* as the gene responsible for m^1G_9 activity (*13*). A library of purified yeast GST-ORF fusion proteins were used to identify the protein responsible for tRNA m^1G_9 methyltransferase activity, Trm10, which was subsequently purified to facilitate characterization of its enzymatic activity, and subsequently shown to be responsible for all known instances of m^1G_9 modification in yeast tRNAs. Importantly, there is no decrease in cell viability resultant from the loss of m^1G_9 as demonstrated by wild-type growth of the deletion strain in rich and minimal media and at different temperatures. This result differs from what is

known about the only other known m^1G forming enzyme in yeast, Trm5, where *trm5*Δ strains grow extremely slowly (*14*). Although *trm10*Δ yeast show no observable phenotype under normal physiological conditions, deletion strains grown in the presence of low concentrations of 5-Fluorouracil (5-FU) that are not inhibitory to the growth of a wild-type strain display a non-viable phenotypic response that is exacerbated at elevated temperatures (see Fig.2) (*15*).

One puzzling feature of Trm10 methyltransferase activity is that only a subset of *S. cerevisiae* tRNAs that contain a G_9 are actually m^1G modified by Trm10 in vivo (*3*). Out of the 22 yeast tRNAs that contain guanosine residues at position 9, at least 10 tRNAs, including $tRNA^{Gly}$ are m^1G modified, while at least 12 other tRNAs, including $tRNA^{Val}$ and $tRNA^{Leu}$, do not contain the m^1G_9 modification in vivo (see Fig.4). Protein and tRNA elements that confer substrate specificity have escaped definition thus far. Direct sequence comparison between in vivo m^1G_9 modified and unmodified tRNAs have failed to reveal sequence determinants for substrate specificity. For example, obvious sequence similarities between unmodified and modified tRNAs do not occur near G_9, within the acceptor stem, or along any portion of these tRNAs. Nor is there a correlation between tRNA size or type of amino acid (i.e. charged, aliphatic, small, acid, base etc…) carried by modified versus unmodified tRNAs. Therefore, the basis for Trm10 recognition of some tRNAs but not others is unknown.

A parallel puzzling feature is that Trm10 shows no conspicuous sequence homology with other tRNA methyltransferases that catalyze similar m^1G formation activities, such as TrmD in bacteria and Trm5 in yeast, which both catalyze m^1G_{37} formation in their respective tRNA substrates. Another mystery surrounding Trm10 is that while Trm10 homologs are conserved throughout yeast and eukaryotes, multiple isoforms of the *TRM10* gene are present in higher order eukaryotes such as *Mus musculus* and *Homo sapiens*. A function for any of the multiple

copies in higher order eukaryotes has not been established, however recent evidence suggests a potential biological role for at least one isoform in *H. sapiens* mitochondria as a component of an unusual protein-only form of the tRNA maturation enzyme RNaseP (see Fig. 3) (*16*). Therefore, not only is the basis for Trm10 substrate recognition unknown, but the molecular basis for Trm10 methyltransferase activity is unknown as well.

Due to the lack of understanding regarding the previously described aspects of tRNA substrate recognition and catalytic activity of this unusual methyltransferase, the goals of this project were to characterize the biochemical function of Trm10, while attempting to address the aforementioned issues. To investigate the molecular basis for Trm10 m^1G_9 methyltransferase activity in *S. cerevisiae* we have used two general approaches, the first being substrate specificity, which was probed using steady-state kinetic characterization with site-specifically labeled tRNA substrates, and secondly, since there is no structural information available for Trm10, potentially catalytic amino acid residues have been probed using single site alanine variants.

Results

***Saccharomyces cervesiae* Trm10 expressed and purified using an *E. coli* expression system.** The investigations of substrate specificity and catalytic activity of *S. cerevisiae* Trm10 pursued in this research, in both cases, requires the utilization of in vitro enzymatic assays for Trm10 methyltransferase activity. This approach necessitates the use of purified Trm10 protein, and to this end, an inducible expression plasmid containing N-terminally His_6-tagged *S. cerevisiae TRM10* with Amp^r marker was transformed into *E. coli* strain BL21(DE3)pLysS. Assessment of the soluble crude extract by SDS-PAGE showed elevated levels of Trm10 expression relative to other proteins, and purification by IMAC followed by dialysis yielded yeast Trm10 that was >90% pure, as judged by SDS-PAGE analysis. Purified wild-type Trm10 was stored at -20 °C and the catalytic activity of the purified enzyme remained stable for at least 6 months upon storage.

Purified Trm10 catalyzes m^1G_9 formation using in vitro transcribed, ^{32}P labeled tRNAs. Based on the observation that Trm10 methylates some, but not all, G residues found at position 9 of yeast tRNAs in vivo, the ability of Trm10 to selectively methylate specific tRNA substrates was investigated. To address this fundamental question, three tRNAs with different methylation patterns observed in vivo were chosen as substrates for these assays: $tRNA^{Gly}$, which contains the m^1G_9 modification in vivo, in addition to $tRNA^{Leu}$ and $tRNA^{Val}$, which contain unmodified G_9 nucleotides in vivo. To assess the biological activity of *S. cerevisiae* Trm10 purified from *E. coli*, Trm10 activity was measured using each of these three tRNA substrates, which were produced by in vitro transcription in the presence of α-^{32}P GTP, resulting in tRNAs that are uniformly radiolabeled at the 5'-phosphate of each G-residue in the tRNA primary sequence. All assays were conducted under standard methyltransferase assay conditions (see

methods) where, in brief, the tRNAs were reacted with Trm10 and the SAM methyl donor, product tRNAs are isolated using PCA (25:24:1 Phenol:Chloroform:Acetic Acid) extraction, tRNA reaction products are digested to 5' monophosphorylated nucleotides with nuclease P1 and subsequently resolved by thin-layer chromatography (TLC) to separate p*G substrate and p*m^1G product.

Using this assay the maximal expected product formed is limited by the fact that only one m^1G nucleotide is formed out of all the radiolabeled G-residues in the tRNA, and thus the maximal percent of product formation can be calculated as 1/n*100%, where n= the total number of guanosine residues in each substrate tRNA (Fig.5 B). This assay showed that $tRNA^{Gly}$ contains an m^1G modified residue after reaction with Trm10, in amounts that correlate with the predicted maximal yield of modification if a single G residue were modified in this tRNA substrate. However there are several issues with this type of assay with regards to further investigation of Trm10 activity. First, use of a uniformly labeled tRNA substrate does not allow for specific determination as to whether this modification occurs specifically at position 9. Second, due to the low sensitivity of this method, equivalent product formation appears to be present in the reactions with similarly labeled $tRNA^{Val}$ and $tRNA^{Leu}$ (Fig.5 A $tRNA^{Val}$ not shown), which could not be reliably distinguished from high levels of background radioactivity in individual reactions. These two considerations need to be addressed by a Trm10 assay that has improved sensitivity for determining m^1G modification selective for position 9. On a similar note, steady-state kinetic analysis would not be possible using uniformly labeled tRNA substrates, because measurement of initial rates of the reaction would require measurement of very low levels of product formation.

Use of site-specifically labeled, in vitro transcribed tRNA substrates show Trm10 catalyzes m^1G_9 formation for $tRNA^{Gly}$ and $tRNA^{Val}$, but not $tRNA^{Leu}$. To address the need for measuring methyltransferase activity with greater sensitivity, an assay using site-specifically labeled tRNA species was performed. These tRNA substrates differ from the uniformly labeled tRNAs in that only the phosphate at position 9 is labeled, and therefore m^1G_9 formation can be specifically observed, and a much broader range of catalytic activity can be measured (up to 100% m^1G_9 tRNA product conversion). Ten fold titration assays of Trm10 with each site-specifically labeled Gly, Val, and Leu tRNA substrate, under the same methyltransferase assay conditions outlined above, was used to determine Trm10 methyltransferase specific activity in vitro (10% m^1G_9 product formation in 2 hours per ug protein; see Fig. 6). Consistent with what is known in vivo, $tRNA^{Gly}$ was a substrate for Trm10 catalyzed m^1G_9 formation, while $tRNA^{Leu}$ did not contain detectable levels of m^1G_9 after treatment with even the highest concentrations of Trm10 achievable in the assay (Fig. 6; Leu data not shown). However, surprisingly, Trm10 methyltransferase assays with the site-specifically labeled $tRNA^{Val}$ showed robust formation of m^1G_9 in this tRNA (Fig.6), a result that is not consistent with the modification status of $tRNA^{Val}$ observed in vivo. Based on specific activity data, quantification of the relative rates of methyltransferase activity with the two Trm10 substrates, $tRNA^{Gly}$ and $tRNA^{Val}$, show that Trm10 is only ~five-fold more active with $tRNA^{Gly}$ compared to $tRNA^{Val}$ (see Fig. 6, Table 1).

Primer extension assay confirms $tRNA^{Val}$ and $tRNA^{Leu}$ contain unmodified G_9 residues in vivo, and that a previously unsequenced species of $tRNA^{Ala}$ contains the m^1G_9 modification in vivo. Given the surprising result that the $tRNA^{Val}$ was a substrate for Trm10 methyltransferase activity in vitro, as demonstrated in the above assay, we decided to explicitly investigate whether, contrary to the available sequence data (*3*), the G_9 residue in this tRNA is

actually modified in vivo. Although 9 tRNA species had been previously shown as subject to Trm10-catalyzed methylation at G_9, the modification status of $tRNA^{Val}$ and $tRNA^{Leu}$ had not been investigated in those assays. Therefore, we used a similar reverse transcriptase (RT)-based primer extension assay, as was used to determine the modification status at G_9 for 9 different tRNAs isolated from *S. cerevisiae* known to contain m^1G_9 in vivo from a previous study (*13*). The identification of m^1G_9 in these previously published assays is based on the observation of a specific primer extension block that occurs at G_{10} if G_9 is *N*-1 methylated, since RT is unable to form the proper Watson-Crick base pair at the m^1G_9 modified nucleotide. To demonstrate that Trm10 catalyzed m^1G_9 formation is responsible for a primer extension block, an RT based primer extension assay was used to compare tRNAs isolated from WT and *trm10*Δ yeast (Fig.7). In this way, the primer extension block should only be observed in tRNA species isolated from wild-type cells, but should not be observed in tRNA species isolated from the *trm10*Δ mutant that does not catalyze m^1G_9 modification. The previous assays were not able to address the modification status at G_9 for $tRNA^{Ala}$, which is known to be m^1G_9 modified in vivo, nor was the in vivo modification status at G_9 looked at for tRNAs used in this study, $tRNA^{Val}$ and $tRNA^{Leu}$. Both of these issues are addressed by results in the current study.

The RT-based primer extension assays were effectively used to analyze the modification status at G_9 for WT versus *trm10* Δ harvested tRNAs for 5 different tRNA species: $tRNA^{Gly}$ and $tRNA^{Trp}$, characterized in the previous study, in addition to $tRNA^{Val}$, $tRNA^{Leu,}$ and $tRNA^{Ala}$, results obtained in this study (Fig. 7). Consistent with in vivo data, wild-type $tRNA^{Gly,}$ $tRNA^{Trp}$, and $tRNA^{Ala}$ all show primer extension stops at G_{10} as a result of the presence of m^1G_9 in the tRNA, while no primer extension stop is observed for these same 3 tRNAs obtained from *trm10*Δ yeast. Also consistent with in vivo sequence data (*3*), $tRNA^{Leu}$ and $tRNA^{Val}$ primers extend

beyond the G_{10} primer extension stop to the 5' terminus of tRNAs harvested from either wild-type and *trm10*Δ yeast, thus these tRNAs do not contain m^1G_9. With respect to $tRNA^{Val}$, this result not only conflates with what had been predicted for its modification status at G_9 in vivo, but also requires the intriguing result that in vitro transcribed $tRNA^{Val}$ is a substrate for m^1G_9 modification by Trm10 despite its lack of modification in yeast.

Determination of steady-state kinetic parameters for Trm10 activity with in vitro tRNA substrates. While the in vitro specific activity measurements indicate only minor differences in the ability of Trm10 to discriminate between $tRNA^{Gly}$ and $tRNA^{Val}$ in vitro, the primer extension data indicate that Trm10 must be able to distinguish between $tRNA^{Gly}$ and $tRNA^{Val}$ in vivo. Therefore it is important to quantify the extent to which Trm10 recognizes certain tRNA species in vitro, in a way that will provide more information about the possible sources for differences in recognition, rather than a simple specific activity measurement. To this end steady-state kinetic analysis was used to determine the parameters k_{cat}, K_M, and more importantly k_{cat}/K_M, which is an indicator of substrate specificity, for the in vitro tRNA substrates, $tRNA^{Gly}$ and $tRNA^{Val}$, with wild type Trm10.

Linear initial rates were measured under saturating conditions for SAM with varying concentration of tRNA substrate, which was in at least five fold excess relative to enzyme concentration. The measurement of linear rates was initially hampered by the observation of severe product inhibition, which necessitated taking many time points early in the expected reaction curve. Notably, this type of product inhibition has also been observed for the TrmD family of enzymes that also catalyze m^1G formation, and may indicate some shared mechanistic features between these two enzyme families that may emerge as the details of the Trm10 catalytic mechanism are revealed. Linear initial velocities, normalized for enzyme

concentration, were plotted against tRNA substrate concentrations in the range of ~0.2 to 4 times K_M (Fig.8) and the resulting data were fit to the Michaelis-Menten equation to yield the kinetic parameters k_{cat}, K_M and k_{cat}/K_M. The Trm10 steady-state kinetic parameters k_{cat}, K_M, and k_{cat}/K_M with $tRNA^{Gly}$ and $tRNA^{Val}$ are very similar (see Fig.8, Table 2). This result reinforces the observation seen with Trm10 specific activity for $tRNA^{Gly}$ and $tRNA^{Val}$ (see Fig. 6 Table 1), and leads to the conclusion that tRNA sequence alone is not a determinant for Trm10 m^1G_9 methyltransferase activity.

We note that these results also constitute the first measurements of steady-state kinetic parameters for Trm10 activity with tRNA substrates, which will also serve as an important foundation for the future analysis of Trm10 variant proteins produced during investigation of the second aim of this research project, described below.

Alanine-scanning mutagenesis to identify amino acids that are potentially important for the *S. cerevisiae* Trm10 catalytic mechanism. Identification of Trm10 residues that participate in the methyltransferase activity is important in order to understand the potentially novel catalytic mechanism of this enzyme family. Moreover, identification of catalytic amino acid residues will be critical to understanding the relationship between Trm10 methyltransferase activity and the unusual 5-FU hypersensitivity of the deletion strain, as well as the participation of Trm10 in the unusual proteinaceous form of RNase P (see discussion). In the absence of structural information or significant homology to other known enzyme families, the method of multiple sequence comparison was used to identify potential targets for investigation, on the basis that amino acids that participate in catalysis are also likely to be highly conserved between Trm10 family members.

To determine amino acid residues necessary for Trm10 methyltransferase activity, two different sequence alignments have been used. The first alignment included an exhaustive sampling of Trm10 homologs across eukaryotes, including all of the multiple Trm10 isoforms observed in higher eukaryotes, such as *H. sapiens* and *M. musculus* (Fig. 9). Alanine-screens, based on this alignment, were used to probe among a candidate pool of 8 highly conserved residues. The variant proteins were expressed and purified as described previously for wild-type Trm10. The yields and purity of the final protein preparations were somewhat variable; the implications of this are discussed in more detail below. For all of the variants, the specific activities with site-specifically labeled $tRNA^{Gly}$ and $tRNA^{Val}$ substrates were measured to characterize the alanine-variants activity relative to wild type Trm10 (Fig. 11 1st 8 residues). Results indicate that two residues, E111A and Q118A, are likely to participate in Trm10 catalysis due to nearly 100 fold decreases in specific activity relative to wild type for both tRNA substrates, $tRNA^{Gly}$ and $tRNA^{Val}$. Other interesting variants include K215A and R243A. We note the potentially interesting roles of K215 and R243, which may play roles in tRNA substrate recognition, since these variants were ~10X fold less active than wild-type Trm10 with $tRNA^{Gly}$, yet only ~2X less active than wild-type Trm10 with $tRNA^{Val}$ (data not shown). However, given the variability in purity for each of the variant proteins, these assays are unable to distinguish between decreases in specific activity that result from overall decreased stability of the variant protein versus decreases that result from direct effects on catalytic activity due to amino acid variation.

Based on complementation experiments in yeast (data not shown and unpublished) and results that indicate an alternative biological role of at least one of the *H. sapiens* Trm10 isoforms, a new alignment was produced that excludes some of the more distantly related Trm10

homologs found in higher eukaryotes, on the basis that additional residues more relevant to m^1G_9 formation would be revealed. As might be expected in this case, several more highly conserved residues were apparent in the new alignment, and 12 of these additional amino acid residues were also investigated by mutagenesis to alanine, revealing 6 more residues that may be catalytically important in the Trm10 methyltransferase reaction; thus bringing the total to 10 residues which may be catalytically relevant (Fig. 11). Based on the second alignment, D100, N126, and W264 appear to be most affected by mutagenesis to alanine, while M107A, Y185A, and Y203A, show significant decreases in activity as well. However, from this set of alanine variants, only N126A seems to be stable, the other 5 variants display significant structural instability. Due to a significant amount of apparent structural instability (as revealed by increased susceptibility to proteolysis compared to the purified wild-type enzyme as well as increased amounts of precipitation of the purified protein during storage after purification) observed in 5 of these 8 alanine variants, the distinction between structurally important versus chemically important residues needs to be elucidated by further purification, or biophysical techniques.

Discussion

S. cerevisiae Trm10 m^1G_9 methyltransferase activity has been investigated using two approaches: characterization of substrate specificity and determination of amino acid residues necessary for catalysis. It has been shown that *E. coli* purified Trm10 catalyzes *N*-1 methylation at G_9 for specific tRNAs; determined by sensitive quantification and comparison of Trm10 methyltransferase (m^1G_9) activity with different site-specifically labeled tRNAs. Determination of steady-state kinetic parameters for unmodified *in vitro* transcripts, $tRNA^{Gly}$ and $tRNA^{Val}$, shows simple nucleotide sequence is *not* the determining factor for Trm10 methyltransferase activity in vitro. In vivo isolated tRNAs from *trm10*Δ yeast lack m^1G_9 containing tRNAs, an observation relevant to future projects in the lab. Finally, at least 10 residues have been identified that could participate in Trm10 methyltransferase activity, however the distinction between direct catalytic importance versus structurally important amino acids residues remains unclear.

On the topic of substrate specificity, the results from this study suggest that the mechanism by which Trm10 recognizes a specific subset of G_9-containing tRNAs for modification is more complicated than might be expected, and similarly raises many new questions to be addressed in future studies. Specifically, the observation that $tRNA^{Gly}$ and $tRNA^{Val}$ are both recognized as Trm10 substrates in vitro, yet the same $tRNA^{Val}$ species is not modified in tRNA isolated from yeast suggests that additional mechanisms must exist to allow Trm10 to recognize substrate versus non-substrate tRNAs in vivo. One possible explanation for this result may stem from the observation that the tRNA substrates for the in vitro assays are also created enzymatically in vitro by T7 RNA polymerase, and therefore lack the full complement of additional modifications present in in vivo produced tRNAs. Therefore, it is possible that

additional modifications are required for the tRNAs to be recognized according to their known in vivo specificities. To test the hypothesis that other tRNA modifications may contribute to substrate specificity in vivo, bulk RNAs isolated with this method will be subjected to Trm10 methyltransferase assay conditions and then probed for modification status using the primer extension assay described previously. Similarly, it appears that Trm10 is able to discriminate against $tRNA^{Leu}$ in vitro, but not $tRNA^{Val}$, raising the possibility of multiple levels for substrate recognition, where some tRNAs (such as $tRNA^{Leu}$) are not inherently accommodated for in the Trm10 active site, while others (such as $tRNA^{Val}$) might be accommodated under certain conditions and yet rejected for modification at a different step. Another possibility related to substrate specificity, may be that protein factors in vivo may act in complex with or independently from Trm10 to determine substrate recognition. Investigation of other tRNA species in terms of their ability to be methylated by Trm10 in vitro, could provide clearer information regarding whether the types of substrate specification mechanisms mentioned are likely observed within yeast, or indeed, other organsisms.

Based on genomic evidence many lower order eukaryotes, including *S. cerevisiae*, contain only one isoform of Trm10, however in higher order eukaryotes like *H.sapiens* there are at least 3 isoforms present (designated Methyltransferase domain-1 MTD1, MTD2, and MTD3). Although all three isoforms share a significant degree of sequence homology, phylogenetic analysis showed that MTD2 is the most homologous to yeast Trm10 (*13*). The presence of multiple isoforms may be closely tied to the questions of substrate specificity that have been addressed by this research project. Do all three isoforms act on the same subset of tRNAs? For instance does one tRNA get modified by MTD1 and another tRNA get modified by a different isoform? Are they modified by combinations of isoforms? Are they localized differently in the

cell to only operate on tRNAs from certain sub-cellular compartments? One way to examine substrate specificity in vivo is to use complementation experiments with Trm10 homologs from a variety of eukaryotes. However, *trm10* knockout yeast do not display an observable phenotype under normal physiological conditions (*13*). Instead, the basis for a screen relies on the recent observation that many tRNA and rRNA modification enzymes are sensitive to 5-fluorouracil (5-FU), with *trm10*Δ yeast being the most sensitive to 5-FU cytotoxicity, and moreover that sensitivity increased with higher growth temperatures (*15*).

We have recently shown that the 5-FU hypersensitivity of *trm10*Δ yeast can be reverted when a wild-type copy of *TRM10* operated under a Galactose-inducible promoter is transformed into yeast deletion strains and grown on YPD+Gal+5-FU (unpublished results). Moreover, only the MTD2 isoform of human Trm10, which is most closely related to yeast Trm10, is capable of reverting the 5-FU hypersensitivity phenotype of the yeast deletion strain. It is intriguing to postulate that this apparent difference in biological function of the multiple isoforms in metazoa may be related to differences in tRNA substrate specificity, and thus one could use a similar model to many of the experiments described in this work to examine the three *H. sapiens* Trm10 isoforms with respect to their substrate specificity.

The *trm10*Δ 5-FU phenotype observation also lays the groundwork for a different set of experiments that utilize the power of yeast genetics. To examine the molecular basis for *trm10*Δ 5-FU sensitivity, a *trm10* mutant gene that encodes for an inactive variant of Trm10, for instance the Q118A variant from this study which is defective for m^1G_9 methyltransferase activity, can be transformed into the *trm10Δ* yeast strain to see if it can revert 5-FU sensitivity. One could imagine that if the 5-FU phenotype were reverted, then methylation status at G_9 is not the determinant for 5-FU sensitivity, and instead a different biological role for Trm10, separate from

methyltransferase activity, is being affected by the presence of 5-FU. The authors of the 5-FU hypersensitivity in *trm10*Δ yeast study hypothesized that the presence of m^1G_9 helps to stabilize specific tRNAs, and thus the observation that 5-FU hypersensitivity was exacerbated at higher temperatures provides evidence that tRNA stability is compromised by the lack of methylation at G_9. Given the scenario that the 5-FU hypersensitivity is not reverted by transformation of an inactive variant into *trm10* knockout yeast, then one could isolate specific tRNAs known to contain m^1G_9 in vivo from wild type and *trm10*Δ yeast to produce melting profiles, where UV absorption would be plotted against temperature to determine if stability of tRNAs are being compromised due to absence of m^1G_9 modification.

In general the cellular locations, and indeed the subnuclear locations, where tRNAs are modified is an area of particular interest for researchers who study tRNA modifications (see review (*17*)). Moreover the cellular locations of certain modification enzymes may differ upon comparison from species to species. For instance a portion of Mod5p-II pool, part of the Mod5p family of enzymes responsible for i^6A_{37} formation in yeast tRNAs, is present in the nucleolus (*18*), while in many budding yeast Mod5p-II is believed to be cytosolic (*19*). Another example of cytosolic tRNA modification enzymes includes *S. cerevisiae* Trm7p, responsible for nucleotide 2'O-ribose methylation in the anticodon loop at nucleotides 32 and 34 (*20*). Also in *S. cerevisiae* Pus1p, which catalyzes formation of Ψ from uridine, is found at the nuclear pore (*21*). To the central point, this survey of tRNA modification enzymes, present in the nucleus, nucleolus, and cytosol, provides a general sampling of the diverse areas tRNA modifications are known to localize within the cell.

As of now there is no evidence for the precise location of *S. cerevisiae* Trm10, or where methyltransferase activity occurs, within the cell; however reconstitution of human

mitochondrial RNaseP (mtRNaseP) holozenzyme identified one isoform of the *H. sapiens* Trm10 homologs (MTD1) as a subunit along with two other proteins (*16*). Unlike all other known RNaseP enzymes, except for spinach chloroplasts and trypanosome mitochondria, this form of human mitochondrial RNaseP is completely proteinaceous and thus does not require a *trans*-acting RNA component to cleave 5' leader sequences during premature mitochondrial, or in the case of spinach, premature chloroplast tRNA processing (*16, 22-24*). It should be noted that there is no evidence for Trm10 homologs present in yeast mitochondria, however this result may explain the biological importance of multiple isoforms in higher order eukaryotes. This observation may also suggest something about where along the tRNA maturation pathway Trm10 m^1G_9 modification occurs, which in turn could also provide fundamental insight into substrate specificity.

In addition to substrate specificity this investigation has also sought to identify amino acid residues that may be necessary for catalysis. We have identified at least 10 residues that could participate in Trm10 methyltransferase activity, 8 that are potentially catalytic and 2 residues that may be involved in substrate specificity. Of the 8 amino acid residues that could be catalytic, five exhibit gross structural instability and need further study to determine if their catalytic importance is a result of effects on chemistry versus structure. The relatively large number of Trm10 alanine variants that exhibit evidence of being structurally destabilized, such as increased susceptibility to proteolysis and precipitation, is unusual with respect to other tRNA-related enzymes that have been studied using an alanine-scanning approach (*25, 26*).

Three of the 8 residues, E111A, Q118A, and N126A, are structurally stable variants that clearly demonstrate chemical importance for Trm10 methyltransferase activity. Although minor structural destabilization resultant from single-site alanine variation can certainly contribute to

slight decreases in activity, it is unlikely to result in 100 fold decreases in activity relative to wild type Trm10 as observed with these variants. Furthermore, two residues may be involved in substrate specifity, K215A and R243A, due to similar specific activities displayed by both variants with respect to wild type Trm10 using $tRNA^{Gly}$ and $tRNA^{Val}$ substrates.

While tRNA modification and processing raises interesting questions and merits future study, SAM-methyltransferases (SAM-MTs) as a superfamily of enzymes are not without their own inherent set of puzzling features; mysteries that can be highlighted using relevant observations with tRNA SAM-MTs. There have been different attempts to characterize SAM-MTs based on a variety of features including secondary structure conservation, domain binding motifs, chemical motifs, and/or amino acid structural motifs. Interestingly, although different families of SAM-MTs share little in the way of identifiable sequence homology, common themes related to overall structural architecture have emerged that suggest distinct evolutionary paths to achieving similar catalytic activities within this family of enzymes. The classic model of SAM-MTs places the SAM binding domain at the N-terminus and substrate binding domain at the C-terminus along a conserved secondary structure architecture of alternating α helices and β sheets (*27*). Interesting families of tRNA SAM-MTs that may share potential features with Trm10, include Trm5 and TrmD, which have a related function to Trm10 in that they catalyze m^1G formation, but each have been classified under two different classes of SAM-MT conserved fold motifs. Close inspection of sequence comparisons between these families of enzymes may provide information related to potential functional or structural similarities at particular sites. However, based on the absence of overall sequence homology between Trm5, TrmD, and Trm10, this otherwise dissimilar family of tRNA SAM-MTs, may suggest, that Trm10 may is representative of even a third class of tRNA SAM-MTs.

Methods

Expression and purification of yeast Trm10. *E. coli* cells were grown in LB+Amp liquid media at 37°C for over-expression of His_6 tagged Trm10, lysed using a French-press and proteins in the resulting soluble crude extract were purified using immobilized metal ion affinity chromatography (IMAC) column directed to the N-terminal His_6 tag in each protein. Fractions of eluted sample were collected from the IMAC column and qualitatively assessed, by visual inspection, for protein content using Bio-rad® protein dye. Fractions that contained the highest protein concentrations were collected and dialyzed into buffer containing 50% glycerol to further concentrate the purified protein samples. SDS-PAGE analysis was used to assess the purity of protein obtained from the column and overall stability of Trm10 purified. Trm10 alanine variants (created as described below) were all expressed and purified in the same way.

Preparation of tRNA substrates. Three different *E. coli* strains, each with Amp^r plasmids that encode for a specific tRNA (either $tRNA^{Gly}$, $tRNA^{Val}$, or $tRNA^{Leu}$) and an upstream T7 RNA polymerase promoter were grown in LB media containing 100 µg/ml Ampicillin, and plasmid DNA was isolated from each culture using commercially available DNA purification kits (Qiagen). The three tRNA encoding plasmids contained BstNI restriction enzyme cleavage sites downstream of the T7 RNA polymerase promoter, which allowed linearization of the isolated plasmid DNA for run-off tRNA transcription with T7 RNA polymerase (purified from a His_6-tagged clone available in the lab). In the transcription reactions, $\alpha^{32}P$ labeled GTP was added to unlabeled GTP in the nucleotide triphosphate (NTP) mix, which results in the transcription of tRNAs that contain ^{32}P labeled guanosines along the entire body of the tRNA wherever a guanosine occurs. The resulting labeled transcripts were purified by denaturing polyacrylamide gel electrophoresis for use in enzymatic assays.

To obtain site-specific, internally labeled tRNA in vitro transcripts, plasmids encoding deletions of nucleotides 1-8 in $tRNA^{Gly}$, $tRNA^{Val}$, and $tRNA^{Leu}$ were created by site-directed mutagenesis and used to transcribe 5' truncated tRNAs. Transcription reactions were the same as described above, except using all unlabeled NTPs, which results in truncated tRNA transcripts that contain G_9 at the 5' terminus. Gel-purified, truncated transcripts were treated with CIP to remove the three 5' phosphates left after transcription and then 5' labeled using T4 polynucleotide kinase and γ^{32}P ATP. The purified, labeled RNA was ligated to a synthetic RNA oligonucleotide (Dharmacon) that recapitulates the 8 missing nucleotides in a DNA-splinted ligation reaction using T4 DNA ligase (*28*). DNA oligo splints complementary to the 5' end of truncated, labeled tRNAs and the synthetic RNA oligo were used to anneal the RNA strands for T4 DNA ligase activity.

Correct ligation of site-specifically labeled tRNA products was verified by digestion with T2 and P1 nucleases (see Fig. 13). The expected nucleotide products, U_8p^* (T2) and p^*G_9 (P1), were identified based on co-migration with unlabeled 5' monophosphorylated nucleotide standards using two different TLC solvent systems (Fig. 13B). Nuclease T2 digested tRNAs resolved in the 0.5M NaFormate TLC solvent system enable accurate quantification of ligation efficiencies based on the ratio of U_8p^* to p^*G_9p, since these two labeled nucleotides migrate independently from one another, as well as other partially digested T2 tRNA products. Fig.XXXX shows ligation efficiencies with respect to Gly, Val, and Leu tRNAs.

In vitro methyltransferase activity assay. For all labeled tRNAs tested, purified Trm10 was added in the indicated amounts to reactions containing ~5000 cpm ^{32}P labeled tRNAs, 0.5 mM SAM, 50 mM tris pH 8.0, 2.5 mM $MgCl_2$, 1 mM DTT, 50 mM NH_4OAc, 1mM spermidine, and reactions were incubated at 30°C. Addition of phenol was used to stop the reactions at

desired times (2 hours for specific activity determination, or at timepoints designed to measure initial rates of product formation in steady-state kinetic assays) and each tRNA product was digested with Nuclease P1 to yield 5' monophosphorylated nucleotides (NMP). Importantly, Nuclease P1 treatment results in 5' monophosphorylated guanosines and 5' monophosphorylated N-1 methyl guanosines, if Trm10 methyltransferase activity has occurred. Thin layer chromatography on cellulose TLC plates was used to resolve the two nucleotide species due to the difference in migration between guanosine and m^1-guanosine nucleotides (see Fig. 6) in an isobutyric acid:NH_4OH:H_2O (66:33:1) solvent system. To visualize the labeled m^1G_9 and G_9 nucleotides phosphorimaging was used to quantify the amount of radioactivity derived from product and substrate, respectively.

Determination of G_9 methylation in vivo using primer extension assay. Low molecular weight RNAs were harvested from wild type and *trm10*Δ yeast using a hot phenol extraction. Bulk RNA preps were treated with a ^{32}P labelled DNA primer complementary to the D arm of specific tRNAs, and primer extension with RT was used to assay modification status at nucleotide G_9.

Site-directed mutagenesis to create Trm10 alanine variants. To create single-site Trm10 alanine variants a Quikchange® mutagenesis kit was used, using sense and antisense DNA primers complementary to the Trm10 expression plasmid that encode the genetic sequence necessary to change an amino acid X codon, into an alanine encoding codon. PCR-based techniques were used to amplify product plasmids, parent plasmids were digested with BstNI, and PCR product plasmids were transformed into *E. coli* (Xl-1 Blue). Plasmids were isolated from single colonies, grown on LB+Amp, and sequenced to confirm X amino acid codon

mutation to alanine. Plasmids positive for alanine variation were transformed into Bl21(DE3)pLysS and Trm10 alanine variants were expressed and purified as described above.

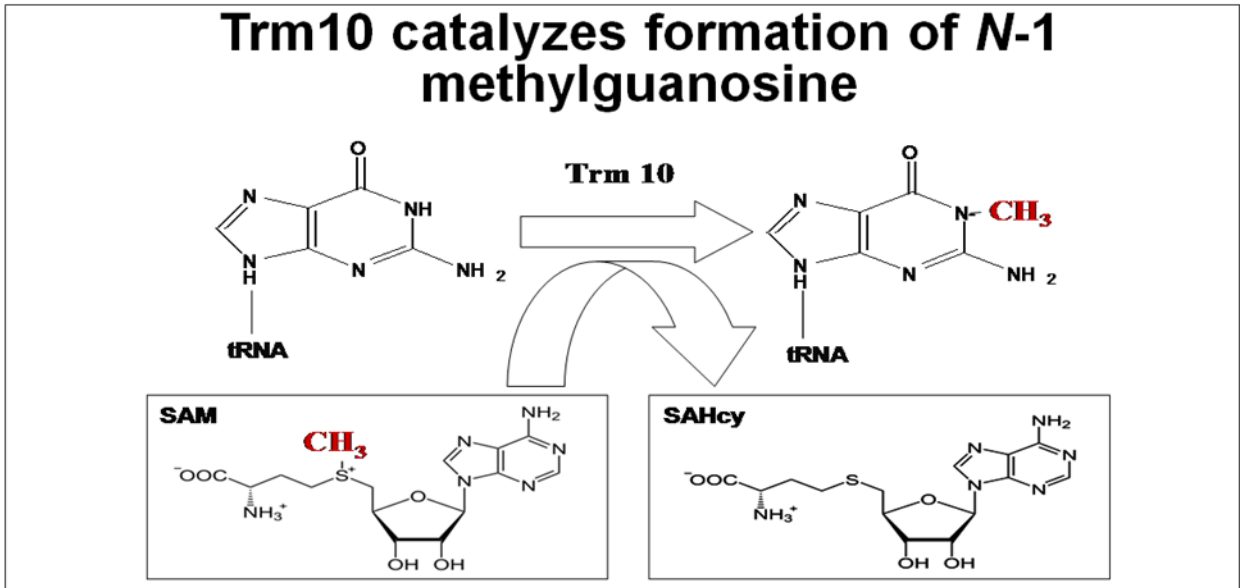
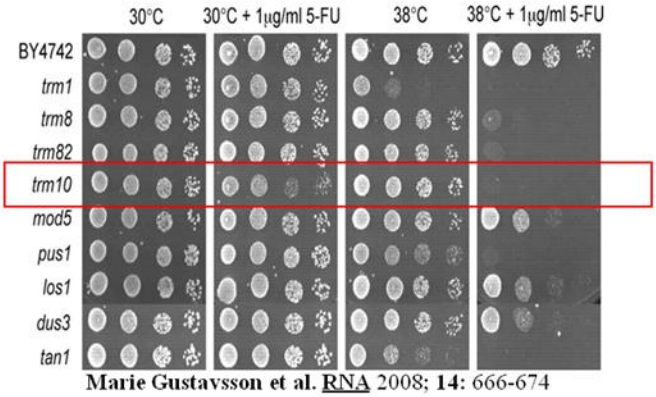


Fig. 1: Trm10 catalyzes *N*-1 methylation of guanosine residues at position 9 for at least 10 tRNA species in *S. cerevisiae*. Trm10 methyltransferase activity proceeds via methylation of guanosine residues at position 9 (m^1G_9) using S-adenosylmethionine (SAM) cofactor as the methyl group donor.

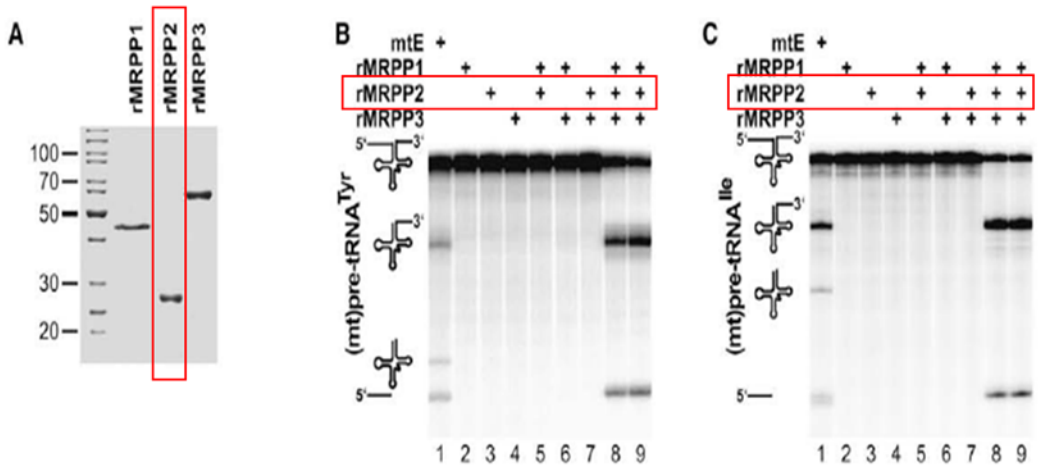
Deletion of Trm10 Causes Hypersensitivity to 5-Fluorouracil (5FU) in yeast



Marie Gustavsson et al. RNA 2008; **14**: 666-674

Fig. 2: Yeast deletion strains, as indicated, were plated on media ± 5FU and allowed to grow at indicated temperature. A genome wide screen for haploid yeast deletion strains sensitive to 5FU show that mutants affecting tRNA maturation were among the most sensitive; *trm10*Δ mutants were the most sensitive.

Reconstitution of Human Mitochondrial RNase P (mtRNase P) Holoenzyme Identified hTrm10 (*H. sap* Trm10 MTD1) as a Subunit



Johann Holzmann et al. Cell 2008; 135 462-474

Fig. 3: A combinatorial purification/ proteomics approach identified 3 protein components, including hTrm10 (rMRPP2), responsible for human mtRNaseP tRNA processing activity. (A) Proteomic characterization of mitochondrial extracts identified three candidate proteins (rMRPP1, rMRPP2, rMRPP3) responsible for human mtRNaseP activity. (B)&(C) Affinity purified candidate proteins were functionally reconstituted to characterize mtRNaseP mitochondrial tRNA processing activity, an enzymatic activity that does not require a *trans*-acting RNA component unlike all known RNaseP enzymes.

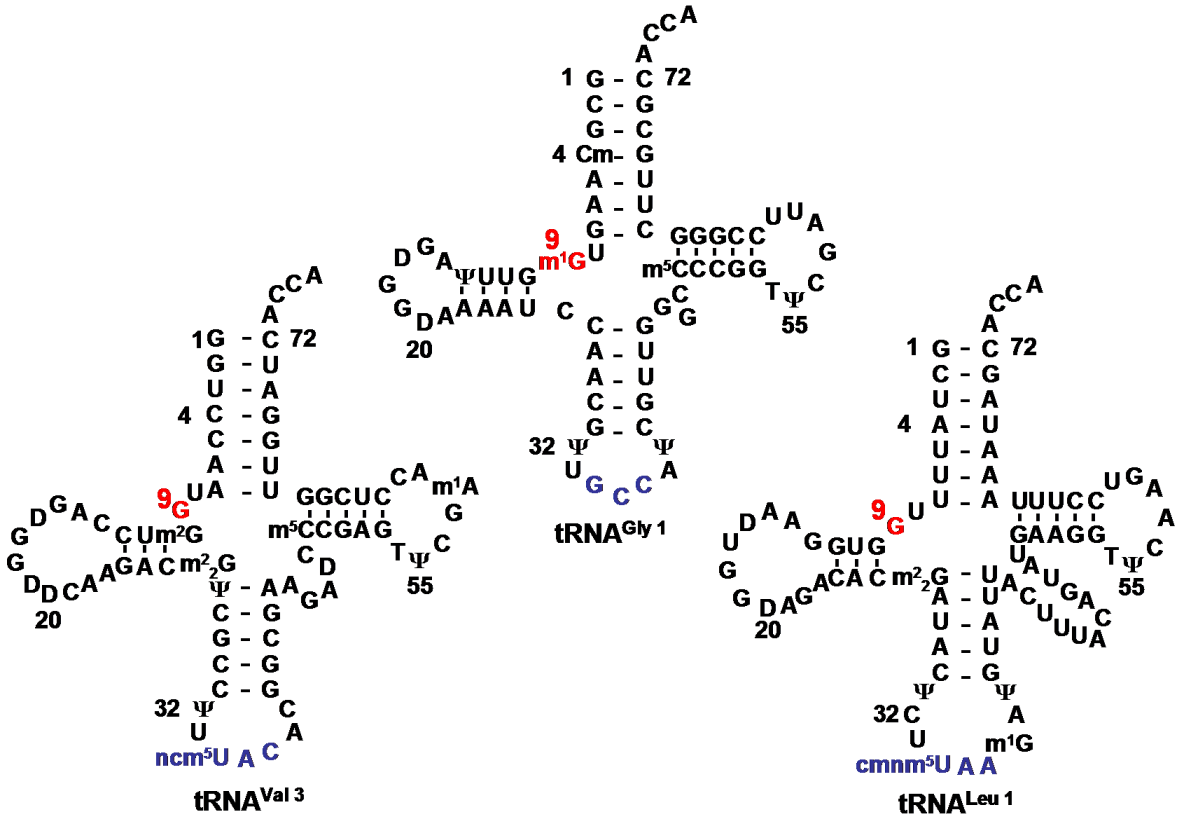


Fig. 4: At least 10 yeast tRNA species contain m^1G_9 in *vivo*, including $tRNA^{Gly}$, while at least 12 others have unmodified G_9 residues, including $tRNA^{Val}$ and $tRNA^{Leu}$. Elements that define tRNA substrates are not obvious from simple sequence comparison.

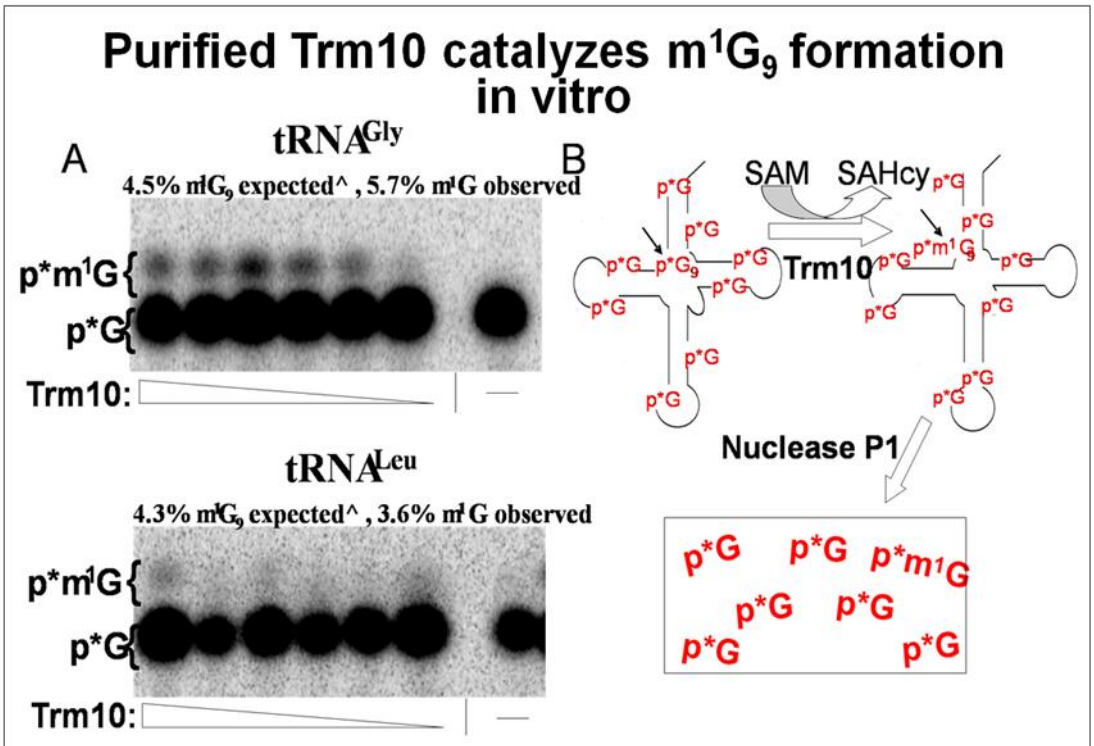


Fig. 5: Trm10 methyltransferase activity assayed using uniformly labeled tRNA^{Gly} and tRNA^{Leu}. (A) Methyltransferase assays contained purified Trm10, 50 mM Tris pH 8, 2.5 mM $MgCl_2$, 1 mM DTT, 50 mM NH_4OAc, 1 mM spermidine, 0.5 mM SAM at 30°C. Reactions were stopped after 2 hrs with phenol, treated with nuclease PI (see B), resolved by thin-layer chromatography (solvent system IBA:H_2O:NH_4OH (66:33:1)), and visualized by phosphorimager. ^Product expected = [1/ (# Guanosine residues in tRNA^{Gly})]*100%. (B) schematic of expected products from nuclease P1 digestion of uniformly labeled tRNA substrates.

Trm10 catalyzes m^1G_9 formation in $tRNA^{Gly}$ and $tRNA^{Val}$ in vitro

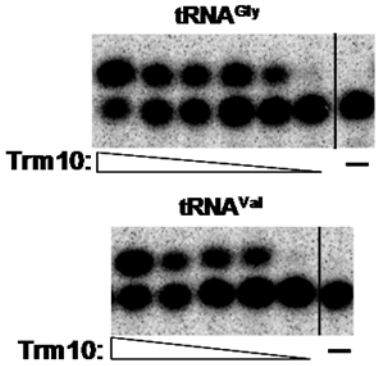


Table I: Relative Specific Activity for m^1G_9 modification by Trm10

Substrate	Specific Activity*
ppp-$tRNA^{Gly}$	25
ppp-$tRNA^{Val}$	5.55

**U/ug protein, 1 U = 10% m^1G_9 in two hours*

Fig. 6: Trm10 activity with site-specifically labeled, in vitro transcribed $tRNA^{Gly}$ and $tRNA^{Val}$, ($tRNA^{Leu}$ not shown). Assay conditions are the same as described in Fig. 5 however, substrates contain 5'-radiolabeled phosphates at position 9.

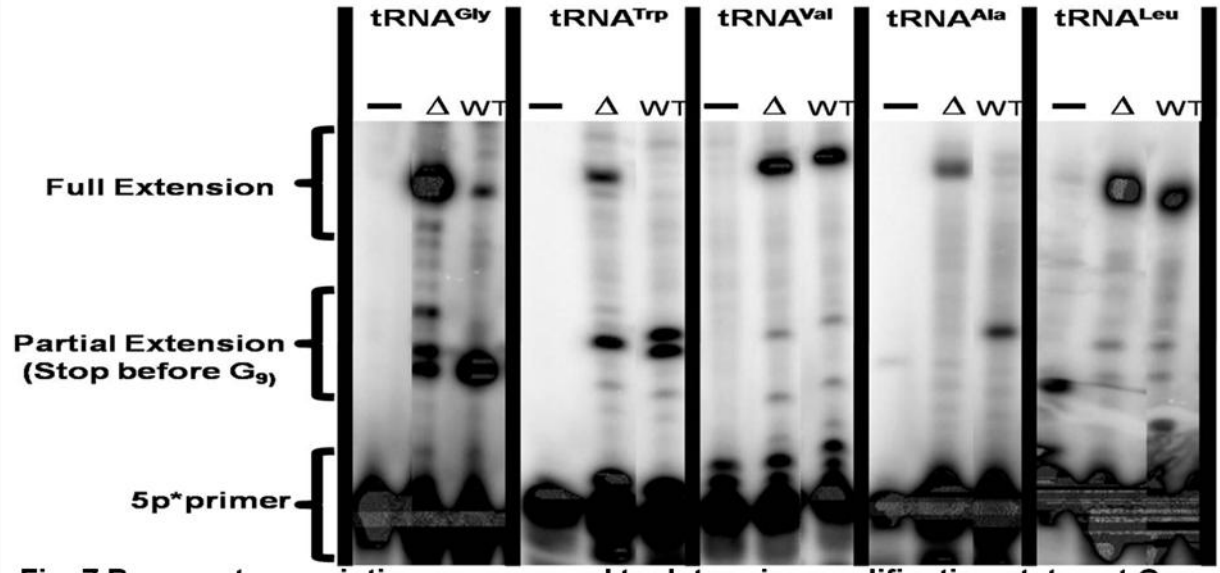


Fig. 7 Reverse transcription assays used to determine modification status at G_9.
Low molecular weight RNAs were isolated from wild-type (**WT**) and *trm10*Δ (Δ) yeast using hot phenol extraction. A previously described primer extension assay was performed with a ^{32}P labeled primer complementary to the D-loop of each indicated tRNA to indicate whether G_9 is N-1 methylated in vivo.

Trm10 Displays Similar Activity with $tRNA^{Gly}$ and $tRNA^{Val}$ in vitro

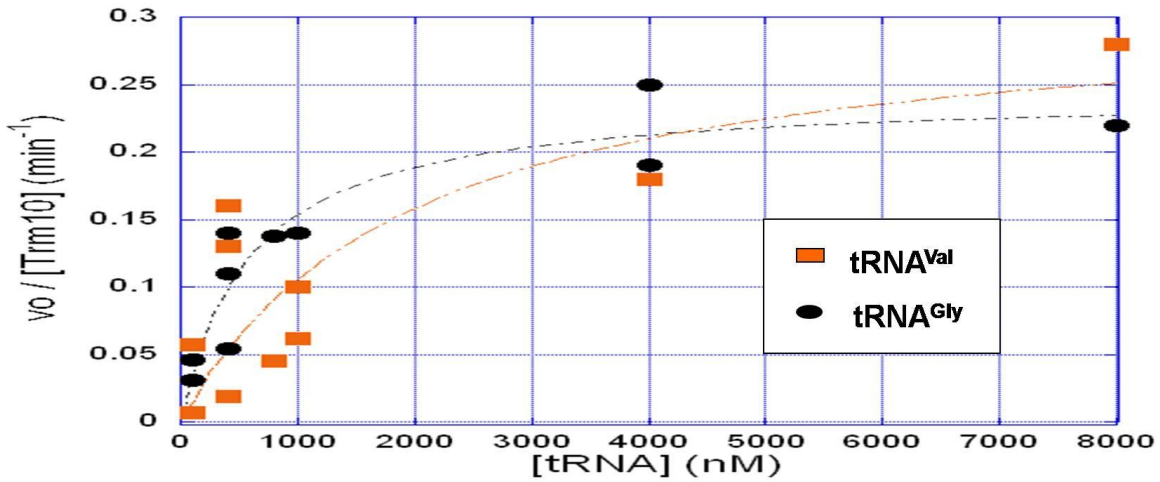


Table 2: Determination of steady state kinetic parameters for $tRNA^{Gly}$ and $tRNA^{Val}$ m^1G_9 modification by Trm10

Substrate	k_{cat} (min^{-1})	K_M (nM)	k_{cat}/K_M (min^{-1} nM^{-1})
ppp-$tRNA^{Gly}$	0.24 ± 0.02	590 ± 180	$4.1x10^{-4} \pm 9.8x10^{-5}$
ppp-$tRNA^{Val}$	0.31 ± 0.09	1945 ± 1350	$1.6x10^{-4} \pm 7.4x10^{-5}$

Fig. 8: Determination of steady-state kinetic parameters for Trm10 activity. Measured initial rates were detemined at varied [S] for each substrate and fit to the Michaelis-Menten equation.

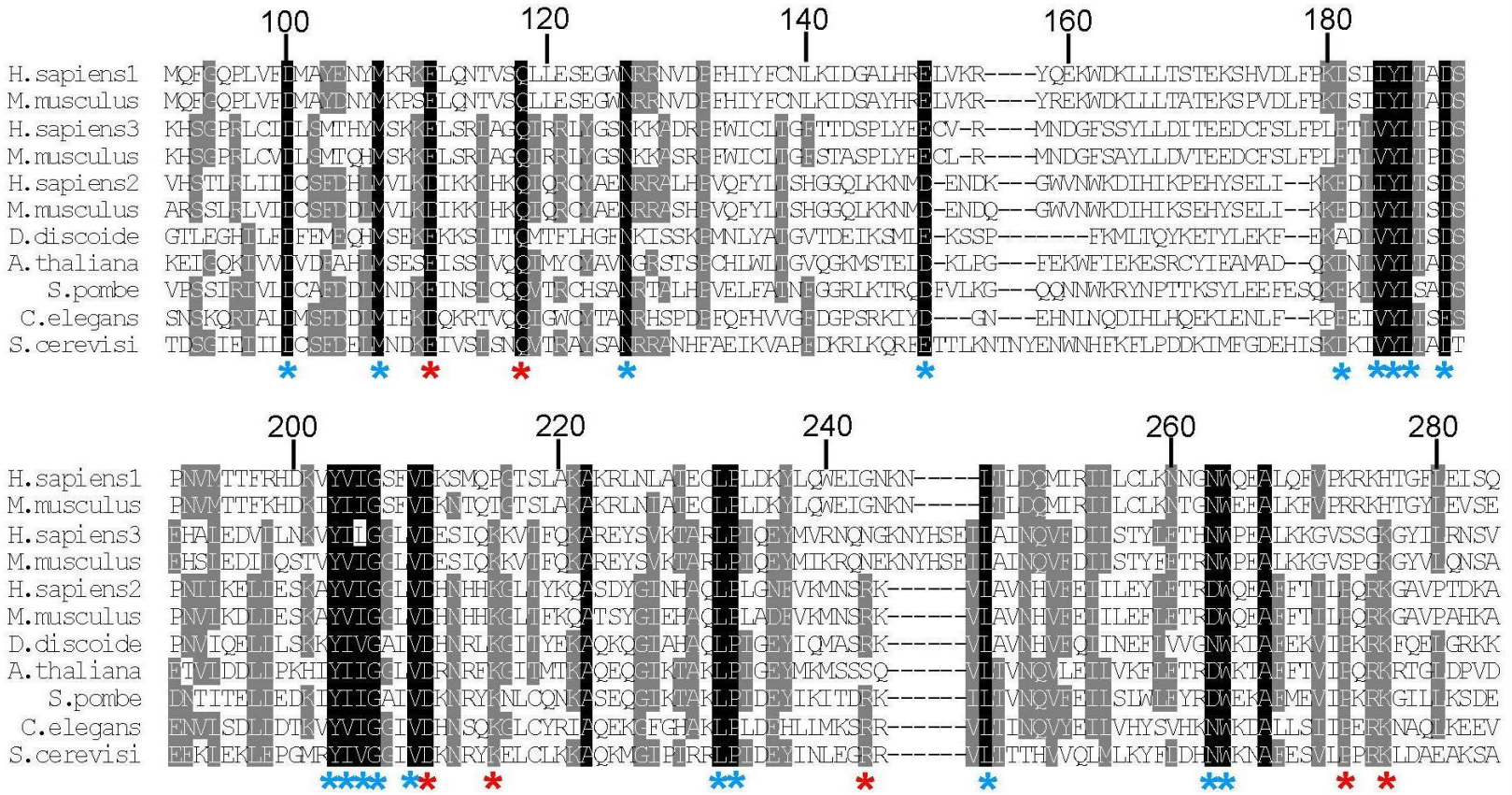


Fig. 9: *S. cerevisiae* Trm10 and its eukaryotic homologs show a limited number of highly conserved amino acid residues. Since Trm10 shows no identifiable sequence homology with other methyltransferases, including the TrmD family (not shown), the Trm10 methyltransfrase catalytic mechanism is likely novel. Asterisks denote yeast Trm10 residues to be studied for participation in catalysis, based on evolutionary conservation, by creation of site-directed variants at the indicated positions. Red: residues investigated in this study, Blue: residues remaining to be investigated.

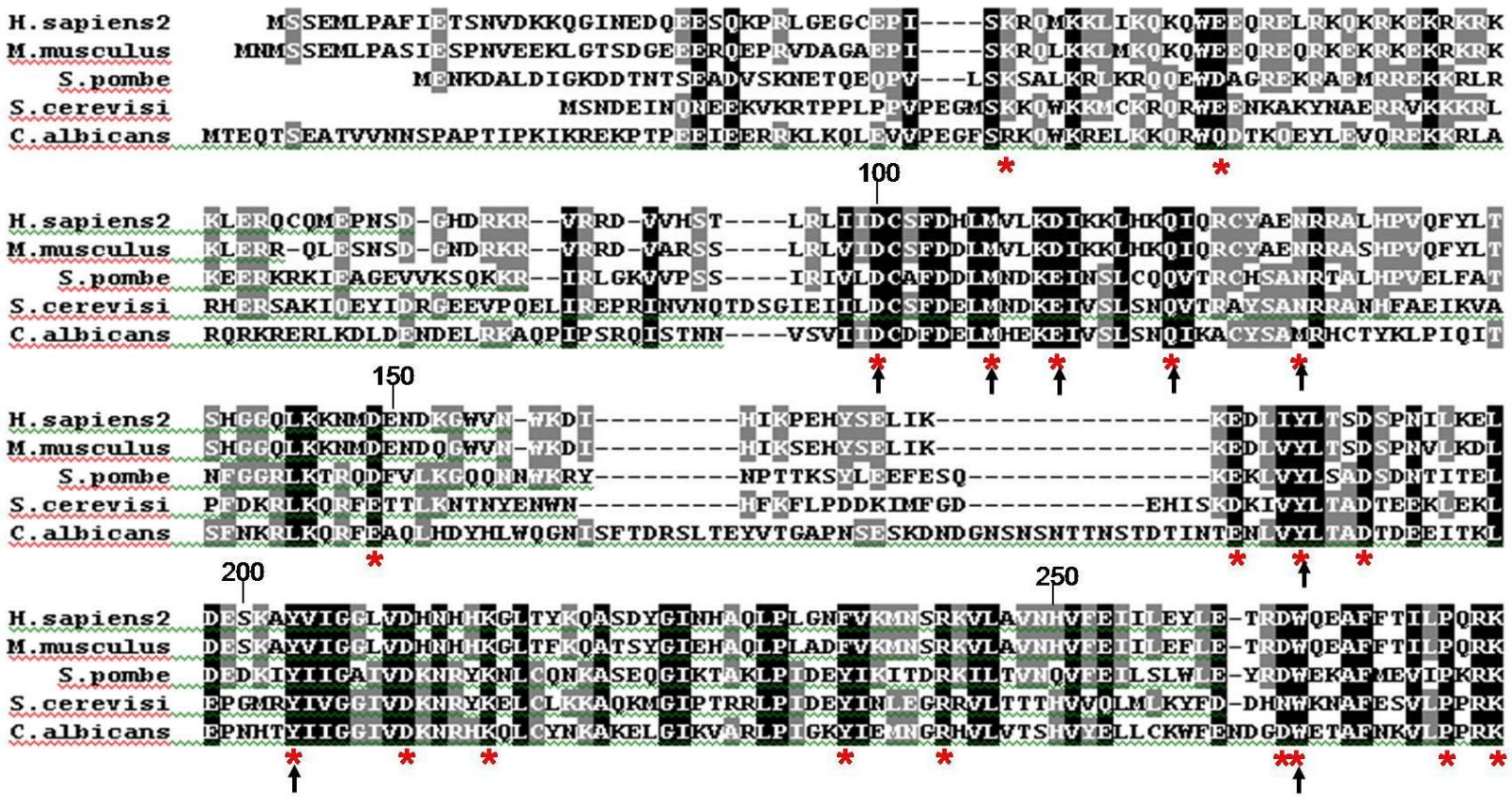


Fig. 10: Multiple sequence alignment of eukaryotic Trm10. Since Trm10 shows no identifiable sequence homology with other methyltransferases, including the TrmD family (not shown), the Trm10 methyltransfrase catalytic mechanism is likely novel. * Denote yeast Trm10 residues studied for participation in catalysis by creation of site-directed alanine variants. ↑ Denote potentially catalytic residues (see Fig. 11 & 12).

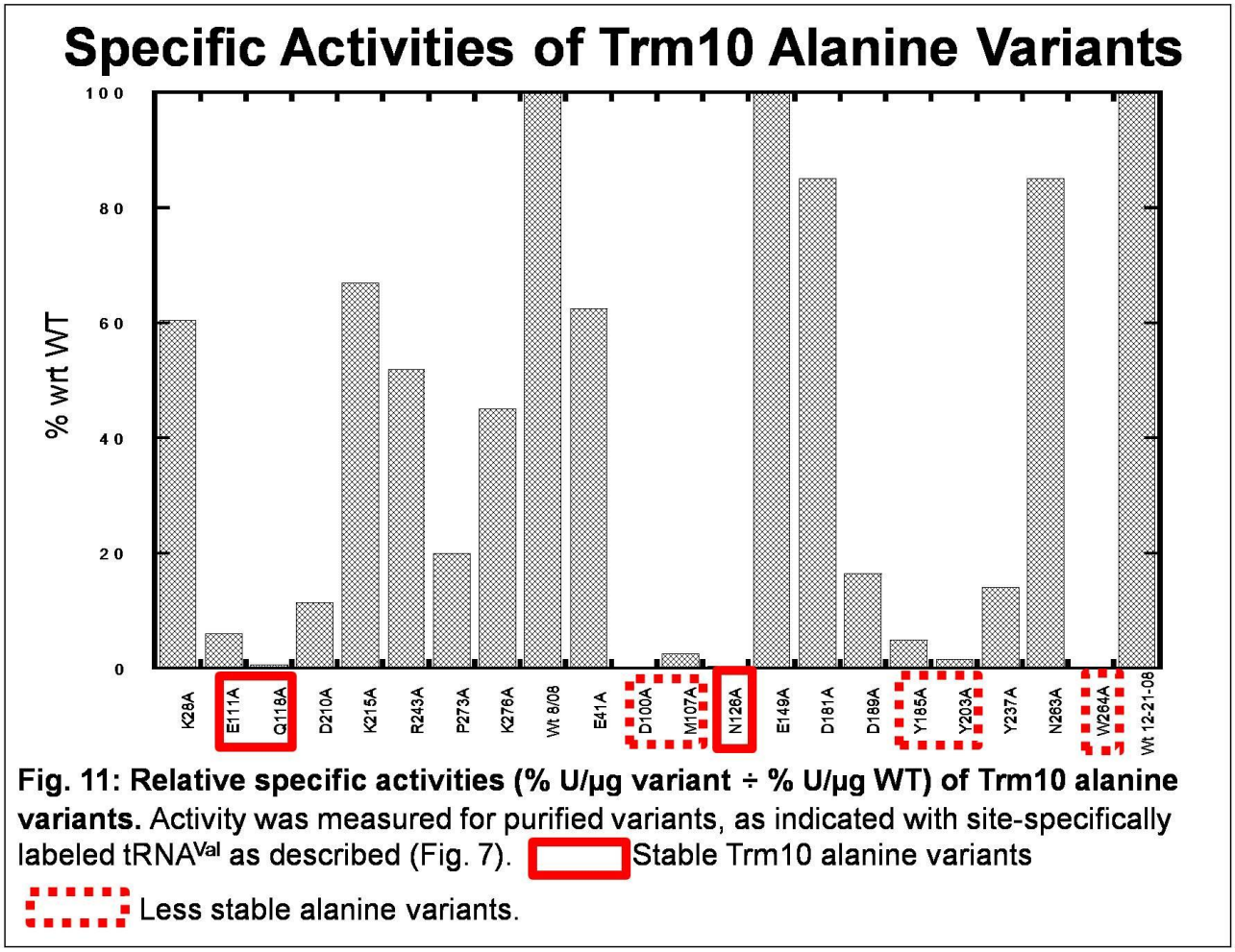


Fig. 11: Relative specific activities (% U/µg variant ÷ % U/µg WT) of Trm10 alanine variants. Activity was measured for purified variants, as indicated with site-specifically labeled $tRNA^{Val}$ as described (Fig. 7). Stable Trm10 alanine variants

Less stable alanine variants.

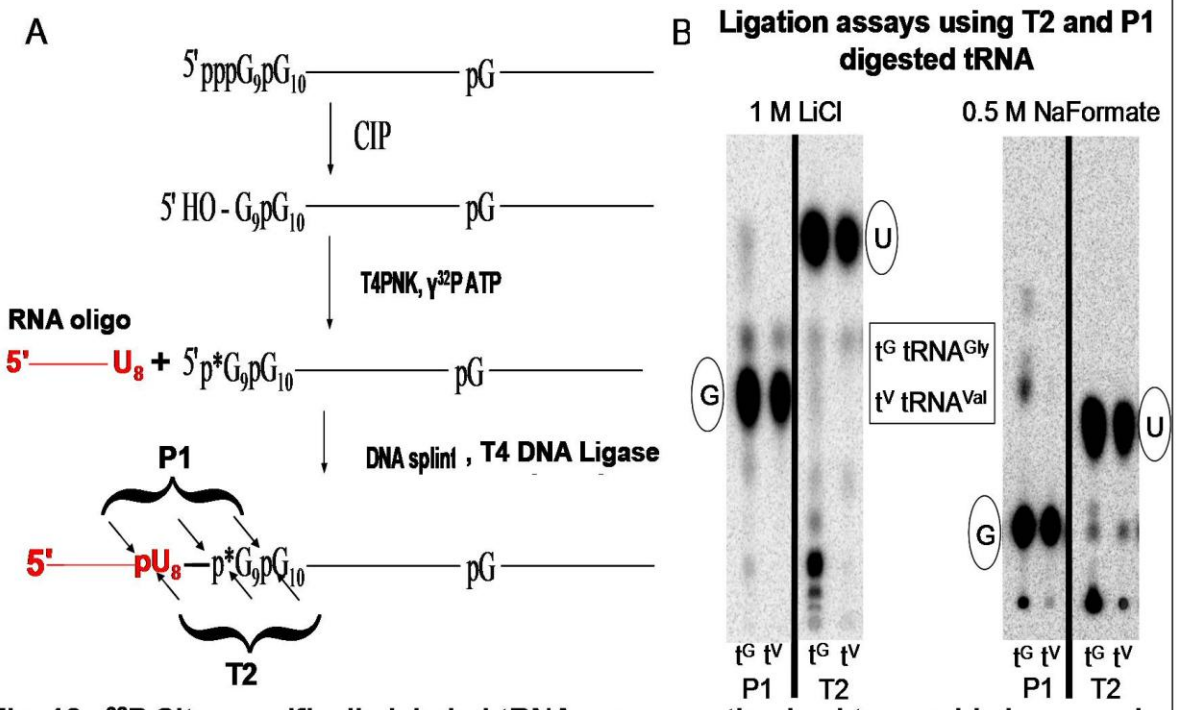


Fig. 12: ^{32}P Site-specifically labeled tRNAs were synthesized to provide increased sensitivity in detection of m^1G_9 product formation by Trm10. (A) Plasmids encoding deletions of nucleotides 1-8 in $tRNA^{Gly}$, $tRNA^{Val}$, and $tRNA^{Leu}$ were created by site-directed mutagenesis and used to transcribe 5' truncated tRNAs. Purified, truncated transcripts were 5' labeled using γ^{32}P ATP. Labeled RNA was ligated to a synthetic RNA oligo containing the 8 missing nucleotides using T4 DNA ligase in a splinted ligation reaction. (B) Correct ligation of products was verified by digestion with T2 and P1 nucleases. The expected nucleotide products, U_8p^* (T2) and p^*G_9 (P1), were identified based on co-migration with unlabeled 5' monophosphorylated nucleotide standards.

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